

Broadly Cross-Reactive, High-Affinity Antibody to Hypervariable Region 1 of the Hepatitis C Virus in Rabbits

Dazhuang Shang, Wenwu Zhai, and Jean-Pierre Allain¹

Division of Transfusion Medicine, Department of Haematology, University of Cambridge, Cambridge, United Kingdom

Received December 24, 1998; returned to author for revision February 10, 1999; accepted March 24, 1999

The HCV hypervariable region 1 (HVR1) of the main E2 envelope protein is critically important in HCV neutralization but its extreme variability makes immune therapy and vaccine development particularly difficult. To explore the hypothesis that HVR1 carries a common epitope susceptible of eliciting cross-reactive neutralizing and inhibitory antibodies, rabbits were immunized with a series of synthetic HVR1 peptides. The anti-HVR1 produced were purified and characterized. Several lines of evidence supported the working hypothesis: (1) although injected only once, a boosting effect from poorly homologous peptides was observed; (2) purified rabbit IgG reacted with high affinity with immunizing peptides and cross-reacted with 16 of 17 unrelated HVR1 peptides; (3) antibodies appeared of restricted diversity irrespective of the linear HVR1 peptide sequences; (4) anti-HVR1 peptides effectively captured HCV in 22 of 33 plasmas from random infected patients; (5) anti-HVR1 IgG blocked the binding of antibody-captured HCV to MOLT-4 cells. These findings suggest that with an appropriate HVR1 peptide immunization scheme, high titer, broadly cross-reactive, blocking antibodies to HCV can be produced. Antibodies to the putative ubiquitous HVR1 epitope may have important clinical uses. © 1999 Academic Press

INTRODUCTION

Hepatitis C virus infection is often persistent because of the inability of the host to mount a sufficiently efficient humoral and cellular immunity (Farci *et al.*, 1992; Weiner *et al.*, 1995; Nelson *et al.*, 1997). This phenomenon is essentially related to the capacity of HCV to escape immune recognition by frequent mutations, particularly in the hypervariable region of the main E2 envelope protein (HVR1) (Weiner *et al.*, 1992; Kato *et al.*, 1993). Random mutations cause amino acid substitutions modifying linear and, possibly, conformational epitopes. Mutants capable of escaping from neutralizing antibodies produced by the patients make immune therapy and vaccine development particularly difficult. The HVR1 domain contains two overlapping linear B-cell epitopes in the C-terminus part that are well exposed at the outside of the virus and appear to be structurally flexible (Kato *et al.*, 1994; Taniguchi *et al.*, 1993). The relatively low level of viremia observed in chronically infected patients may play a role in the poor level of immune response to HCV antigens. Several studies conducted in chronically infected chimpanzees showed that the immune response was unable to protect the animals against reinfection by a different, or the same, viral strain (Farci *et al.*, 1992; Kato *et al.*, 1994; Taniguchi *et al.*, 1993; Okamoto *et al.*, 1992; Farci *et al.*, 1994).

However, several investigators, including our group, observed that the antibodies present in a large proportion of infected patients had significant cross-reactivity with apparently unrelated HVR1 sequences presented as synthetic peptides (Rosa *et al.*, 1996; van Doorn *et al.*, 1995). The observations (1) that three conserved amino acid were present at position 2 (threonine), 23 (glycine), and 26 (asparagine) of the HVR1 sequence; (2) that the extent of amino acid substitution of the C-terminus portion of HVR1 was limited; and (3) that HVR1 was a main site for neutralization playing an important functional role in viral attachment to target cells suggested that HVR1 was likely to present common motifs recognisable by putative target cell receptors.

The present study aimed at gathering evidence that a common epitope was present in the HVR1 region that could be used to develop highly cross-reactive antibodies with sufficient functional potency for diagnostic and, potentially, clinical usage. High titers anti-HVR1 raised in rabbits serially immunized with different HVR1 peptides were characterized for affinity, specificity, and cross-reactivity for HVR1. The ability of these polyclonal antibodies to capture viral particles and to block HCV binding to lymphocytic target cells was studied.

RESULTS

Reactivity of rabbit sera to HVR1 immunizing peptides

Two rabbits were immunized with six individual KLH-conjugated HVR1 peptides (Table 1A). These peptides had between 43 and 71% homology and were selected

¹ To whom correspondence and reprint requests should be addressed at Division of Transfusion Medicine, University of Cambridge, East Anglia Blood Centre, Long Road, Cambridge, CB2 2PT, UK. Fax 44-(0)1223 242044. E-mail: jpa.1000@cam.ac.uk.

TABLE 1
Sequences of HCV Peptides Used in the Study

Peptides ^a	Sequence	Region
A	C K G L N G L F D L G P K Q K I	H V R 1
M H 3	- Y - - T T - - - P - - R - Q -	
M H 4	- S - - S S - - - T P - - - Q -	
L 1 1	C T T S - - A S - - N S - A R - H	
W 1	- D T A - - A - - N - - - - T	
L V	- N T H - I A S - - A F - - A - -	
B		
M H 1	- N R - G - - - N F - - - - -	
M H 5	- N R F A - M - S - - A R - - -	
D H 1	- G - V A - - - K M - S Q - - L	
D H 2	- G M F T - - - N Q - A Q - - L	
U S 1	- S - F V - - - S P - - S - R -	
L B 1	- - T S - F A S - - K F - - S - -	
L B 2	- - T S - F T S - - R - - - S - -	
W 2	- D T S A - V - - L S P -	
F R 1	- N T Y - - T A - L S P - - S - Q	
F R 2	- I V N R R T S F - N - - - S - R	
L 1 2	- - T R - - A S - - T S - A R - H	
S 6 5	- R R V A S F L I R - S A - - -	
S 6 6	- L - I A S F L I R - - - - N -	
S 8 2	- T - F S N - - N - - S Q - - V	
S 8 5	- A A - A S - - L S - - Q - - N	
S 8	- T - F S K H N N M - S Q - - V	
S 9 0	- A A - A N S L S - - - Q - - V	
C		
S 5	A T R K T S E R S Q P R G R R Q P I C	C O R E
S 6	P K A R Q P E G R A W A Q P G -	
S 8	S R P S W G P T D P R R R S R N L G -	
S 1 9	S G H R M A W D M M M N W S P -	E 1
S 3 4	D C F R K H P E A T Y T K C G S G P -	E 2
S 9 1	C G T Y V T G G A S G R T V H R	H V R 1 N

^a Synthetic peptide sequences (using one-letter codes) from HVR1 and non-HVR1 regions of HCV. (A) Peptides derived from the HVR1 C-terminus of HCV isolates used for immunization. (B) Peptides of the HVR1 C-terminus derived from different HCV isolates used for testing the cross-reactivity of antibodies from the immunized rabbits. HVR1 peptides are aligned using MH2 as lead sequence. (C) Peptides S5, S6, and S8 are derived from the core region of the same HCV isolate, S19 from the E1 region, S34 from the E2 region (not HVR1), S91 from the HVR1 N-terminus. These peptides were used for testing the specificity of antibodies from the immunized rabbits. To all peptide sequences a N- or C-terminal cysteine was added to facilitate coupling to the BSA or KLH carriers.

because of a relatively high frequency of cross-reactivity with a previously described panel of HCV-infected patient sera (Jackson *et al.*, 1997). The rabbit antibody response to immunizing HVR1 peptides was tested by ELISA. In rabbit 1, the antibody response to the first three immunizing peptides (MH2, MH3, and MH4) was high and persistent. The antibody response to the next two peptides (W1 and L1.1) was low and was undetectable with the last immunizing peptide (LV) (Fig. 1a). In rabbit 2, antibody response to all immunizing HVR1 peptides was observed. One week after day 45, when peptide MH4 was injected, a high anti-MH4 response was observed and, when peptide MH3 was injected (day 60), a high level of reactivity to MH3 was already present in the rabbit serum. In addition, at day 75, when peptide MH2 was injected, maximum anti-MH2 reactivity was already

reached. These results suggest that some peptides had a clear boosting effect on the production of highly cross-reactive antibodies elicited by a previously injected peptide, although the linear sequences were poorly homologous (Fig. 1b).

Specificity of individual rabbit sera against HVR1 peptides

As shown in Fig. 2, rabbit 1 and 2 sera reacted with several but often different, unselected HVR1 peptides derived from unrelated HCV-infected patients (Table 1). Reactivity against all studied HVR1 peptides except peptide W2 was observed with either or both rabbit 1 or 2 sera. To obtain an antibody with the largest possible HVR1 cross-reactivity, sera collected 90 days post first

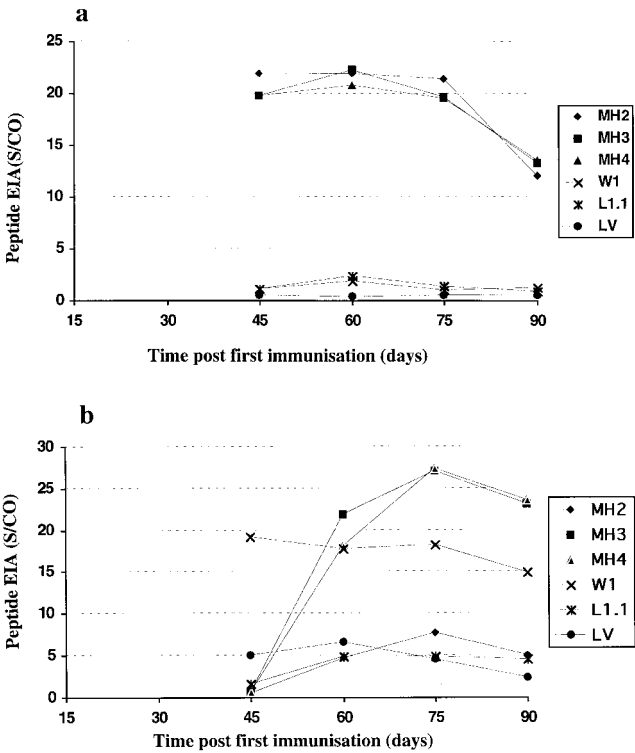


FIG. 1. (a) Immune response of rabbit 1 to 6 HVR1 peptides expressed as sample to cutoff (S/CO) ratio obtained with individual peptide ELISAs. Each rabbit was immunized with a single intradermal injection of 400 μ g of KLH-conjugated HVR1 peptides with Freud's adjuvant (Table 1). Serum samples were taken at 15-day intervals starting at day 45, prior to injection of the third peptide. The mean of 10 replicates of preimmunization plasma plus 4 SD defined the cutoff. Peptide codes are shown in the box; peptide sequences are provided in Table 2. (b) Immune response of rabbit 2 to six HVR1 peptides as in (a) but injected at 2-week intervals in the reverse order. Preimmunization plasma from rabbit 2 was used as a negative control to define the cutoff as in (a).

immunization from rabbits 1 and 2 were mixed and purified IgG was used as a single reagent in further studies (rabbit IgG).

Characterization of rabbit anti-HVR1 IgG

Rabbit IgG concentration was 4.4 mg/ml. Affinity constants against HVR1 representative immunizing peptides MH2, MH3, and LV determined by surface plasmon resonance were K_D s of 2.15×10^{-8} , 6.98×10^{-7} , and 4.79×10^{-6} , respectively (Table 2).

Rabbit IgG titers against HVR1 immunizing peptides, unselected HVR1 peptides, and HCV core, E1, and E2 (other than C-terminus HVR1) peptides were determined by limiting dilution tested by ELISA. As shown in Fig. 3, rabbit IgG reacted with all immunizing peptides and cross-reacted with 16 of 17 unrelated C-terminus HVR1 peptides, with titers ranging between 1:1000 and 1:100,000. No reactivity was observed with five non-HVR1 peptides shown in Table 1c [S5, S6, and S8 (core); S19 (E1); S34 (E2); and S91 (HVR1 N-terminal peptide)].

To examine the potential clonal restriction of the anti-HVR1 reactivity we immunopurified rabbit IgG with two immobilized immunizing peptides (MH3 and W1) and three cross-reactive peptides (S90, LB1, and DH1) and tested the reactivity of these peptide-immunopurified antibodies against our panel of HVR1 peptides and the core S5 peptide as negative control. With each antibody, 12–15 peptides showed a reactivity above the cutoff level (mean of 24 replicate of negative controls plus 4 SD). Twelve peptides reacted with either all (MH3, MH4, W1, S66, MH2, MH1) or 4 immunopurified antibodies (S90, L1.1, LV, US1, FR2, FR1) with sample to cutoff ratios ranging from 1 to 19. When the sequences of these 21 peptides were compared to the sequences of the 11 less or not (W2, S82, S85, S87) reactive peptides, no relationship between the linear peptide sequence and ELISA reactivity was found. Phylogenetic analysis of the 23 HVR1 peptides distributed into two main branches (data not shown). Six of the 12 highly reactive peptides were located in each of these two branches.

To examine the potential importance of peptide conformation in their interaction with rabbit IgG, the 6 immunizing peptides and 11 unrelated peptides (Table 1) were denatured by heating at 100°C for 10 min and used for coating microtiter plates in parallel with untreated peptides. By ELISA, the reactivity of immunizing treated peptides was decreased (mean decrease 17%, range 3–43%). In contrast, reactivity with other peptides was unchanged (8 peptides), decreased (5 peptides, range 6–29% decrease), or increased (4 peptides, range 14–32%).

HCV RNA capture with rabbit anti-HVR1 IgG

Since rabbit IgG had a high titer, high affinity, and broad HVR1 cross-reactivity, we next assessed the capacity of rabbit IgG to capture HCV in plasma, presumably through the HVR1 C-terminus epitope. HCV RNA from 22 of 34 random HCV RNA-positive plasma samples (65%) was detected by rabbit IgG capture assay. Representative examples are shown in Fig. 4. In all cases the plasma supernatant after capture was positive for HCV RNA, suggesting that, when positive, only part of the circulating HCV population was detected with the rabbit IgG-based capture system. The same experiment performed with preimmunisation rabbit antibody mixture was consistently negative.

In order to explore the potential effect of viral concentration on the anti-HVR1 viral capture system, we quantified by limiting dilution of HCV RNA levels of 23 of 34 HCV RNA-positive samples and examined the distribution of positive and negative capture results. As shown in Table 3, the distribution of anti-HVR1 capture positive and negative samples was not significantly influenced by HCV viremia levels within a range of 3×10^3 to 5.2×10^6 geq/mL.

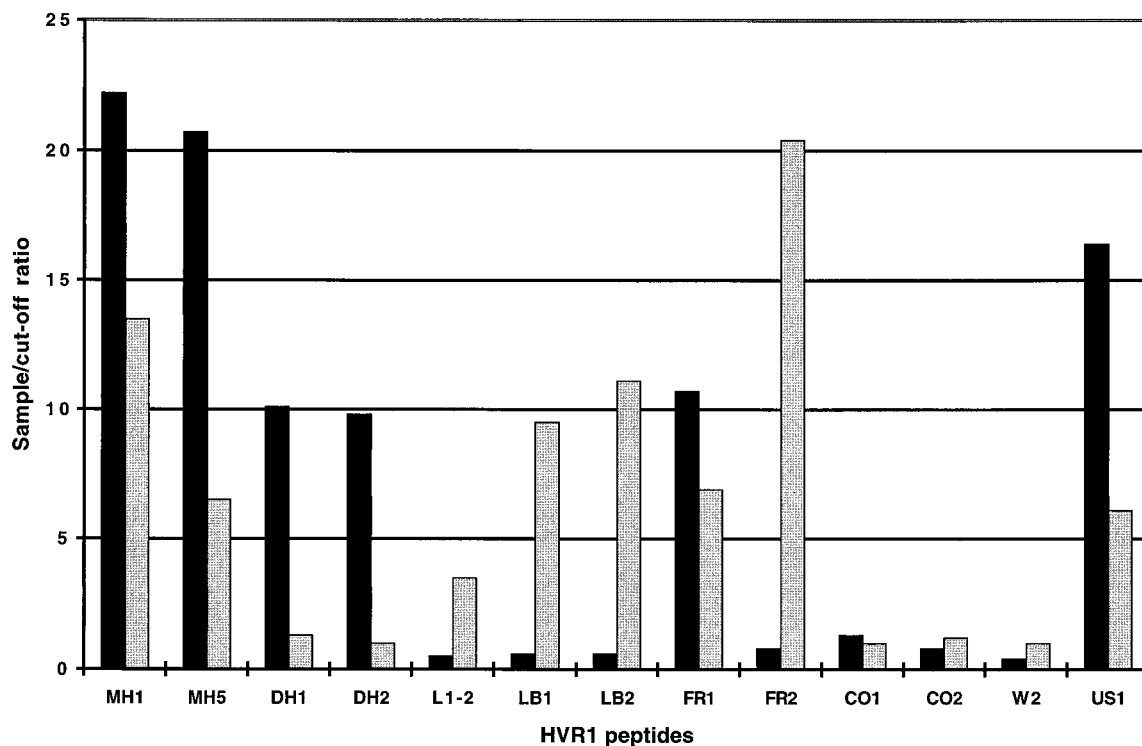


FIG. 2. Cross-reactivity of plasma from rabbit 1 (black columns) and rabbit 2 (gray columns) with 11 HVR1 peptides different from the immunizing peptides expressed as sample/cutoff ratio. Sequences are shown in Table 1. The cutoff of the peptide ELISA was as defined in Fig. 1, using the corresponding preimmunization rabbit plasma. Only peptide W2 did not cross-react with the plasma of either rabbit.

The potential impact of HCV genotype on HCV capture by rabbit antibody was also explored. Twenty-eight available samples were typed with the Innogenetics assay (Petrik *et al.*, 1997). Seven were genotype 1, 5 1a, 9 1b, 2 genotype 2, and 2 2a, and 3 genotype 3a. The rabbit IgG capture assay was positive in 17 of 21 samples, with genotype 1, 1/4 with genotype 2, and 2/3 with genotype 3. The difference in capture positivity between genotypes was not significant.

To determine the mechanisms underlying HCV capture with rabbit IgG, a series of experiments was conducted. Sera from two random capture-positive and two capture-negative sera were submitted to protein G chromatography. Flow-through and eluted fractions were tested for HCV RNA by both rabbit IgG capture and standard HCV RNA detection assay. The standard assay detected HCV RNA in both fractions of all four samples, suggesting that all samples contained free and com-

plexed HCV. The capture assay detected HCV RNA in the flow-through and eluted fractions of capture-positive samples (data not shown). These results suggested that rabbit IgG capture is limited not by the free or complexed state of HCV but by the presentation of the HVR1 epitope. This conclusion was further supported by comparing available HVR1 sequences from three patients: two positive and one negative with the capture assay. The two captured viruses had HVR1 sequences 73 and 66% homologous to the consensus sequence of the six peptides used for rabbit immunization, while the noncaptured virus had 33% homology. Since some of the HCV captured in the eluted fraction was likely to have dissociated during the elution process, we could not determine whether complexed virus was captured. We then ultracentrifuged at 35,000 *g* for 4 h two capture-positive sera and tested HCV RNA by capture and standard assays in the pellet containing mostly complexed virus and the top fraction containing mostly free virus (Hijikata *et al.*, 1993). HCV RNA was detected by capture assay in both pellet and top fractions of the ultracentrifugation tube, suggesting that free and complexed HCV were detectable by rabbit IgG capture. However, when each of these fractions was submitted to protein G chromatography as described above, a strong HCV RNA signal with standard RT-PCR was observed with the top fraction but a weak signal with the bottom fraction from both rabbit capture-

TABLE 2

Affinity Constant (K_d) of Rabbit IgG to HCV HVR1 Peptides

Peptide	K_{diss} ($S^{-1} \times 10^{-3}$)	K_{ass} ($m^{-1}S^{-1} \times 10^4$)	K_d (M)
MH2	1.942	0.901	2.15×10^{-8}
MH3	7.189	1.031	6.98×10^{-7}
LV	5.345	0.111	4.76×10^{-6}

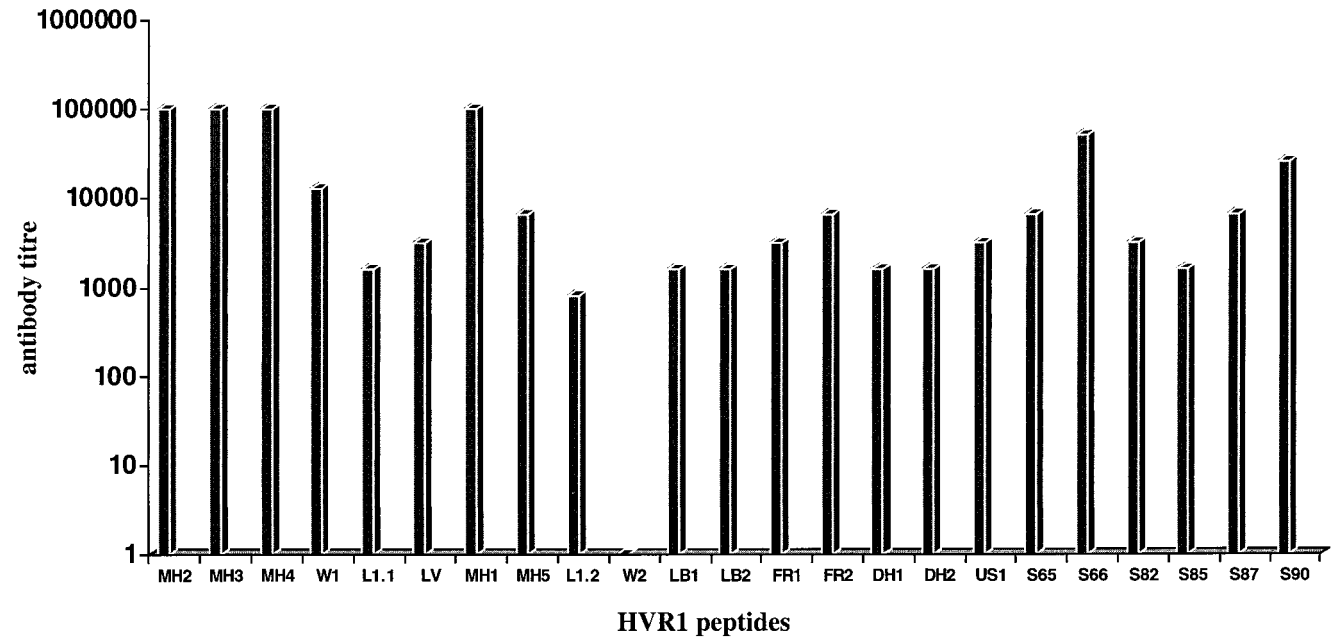


FIG. 3. Titration of rabbit IgG reactivity with six immunizing HVR1 peptides (MH2–LV, first six bars from left) or cross-reactivity with 17 nonimmunizing peptides by ELISA. The titer was calculated as the dilution corresponding to extinction of signal, using preimmunization rabbit IgG mixture to define a negative reaction. Only peptide W2, as in Fig. 2, did not cross-react with rabbit IgG.

positive and -negative samples, suggesting that either some complexed HCV easily dissociated or that the pellet contained small amounts of contaminating free HCV.

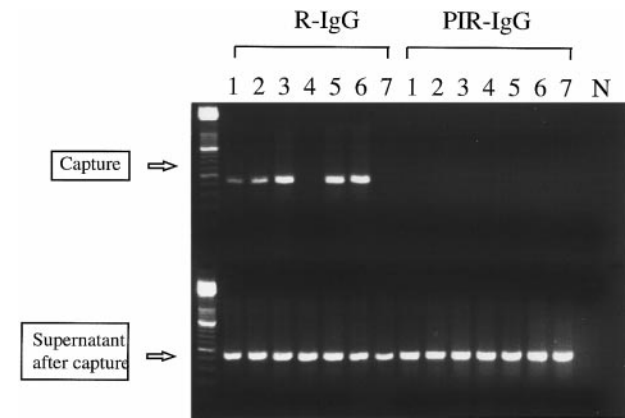


FIG. 4. HCV RNA detection by capture with anti-HVR1 rabbit IgG bound to magnetic microparticles. Representative samples were tested as described under Materials and Methods. R-IgG, rabbit anti-HVR1 IgG used for HCV capture; PIR-IgG, preimmunization rabbit IgG used as negative control. Numbers correspond to the patients' serum sample codes or negative sample (N) controls. The top line shows ethidium bromide-visualized amplicons obtained by nested RT-PCR using core region primers after RNA extraction from washed microparticles. The bottom line shows HCV amplicons obtained after RNA extraction from serum supernatants after HCV capture with microparticles which were used as positive controls. Samples from patients 1–3, 5, and 6 are capture positive; patient 4 and 7 plasmas are capture negative.

Blocking of viral attachment by rabbit anti-HVR1 IgG

The ability of rabbit IgG to block HCV binding to susceptible cells was tested using MOLT-4 cells as target. Plasmas from three patients whose virus was captured by rabbit IgG and two who did not were used as source of HCV. As shown in Fig. 5, HCV binding to MOLT-4 cells was blocked in a dose-related fashion by rabbit IgG only with the three plasmas positive in the HCV capture assay. Partial blocking was observed with 0.5 $\mu\text{g/ml}$ of rabbit IgG and no cell-associated HCV RNA was detectable after incubation of patients plasmas with 5 $\mu\text{g/ml}$ of antibody. Rabbit IgG did not affect HCV binding to MOLT-4 cells when HCV RNA containing plasmas, negative with the HCV RNA capture assay, were used as source of HCV.

DISCUSSION

Mechanisms of HCV persistence include the ability of the virus to mutate, particularly in the neutralizing HVR1

TABLE 3					
Influence of Viral Concentration on HCV Capture					
HCV concentration (geq/ml)	<10 ⁴	10 ⁴ –10 ⁵	10 ⁵ –10 ⁶	>10 ⁶	Total (%)
No. of samples	3	11	2	7	23
Capture positive	2	8	1	4	15 (65)
Capture negative	1	3	1	3	8 (35)

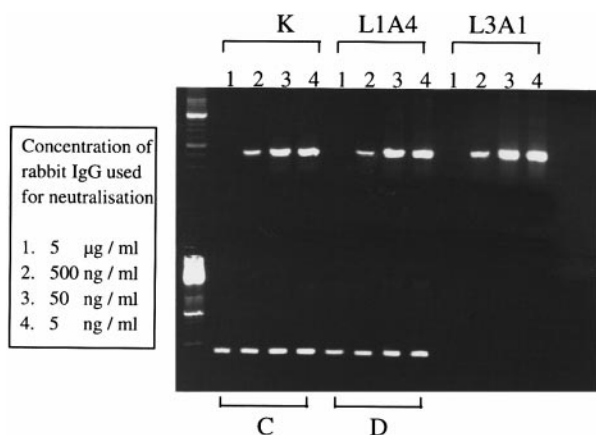


FIG. 5. Effect of preincubation of HCV RNA-positive plasmas either anti-HVR1 capture positive (K, L1A4, L3A1) or negative (D, C) with four concentrations of rabbit IgG (1–4 or 5 μ g–5 ng/ml) on the HCV binding to MOLT-4 cells. The figure is representative of experiments performed in triplicate. Negative controls were cells in plasma without HCV; positive controls were supernatants of the incubation mixture. In all three HCV capture-positive samples, 5 μ g/ml of rabbit IgG totally blocked HCV binding; partial blocking is obtained with 0.5 μ g/ml. No blocking is observed with two HCV capture-negative samples.

region of E2, the relatively low immune reactivity of HCV proteins (in part related to the low level of viremia), the high specificity of antibodies to HVR1 which does not cross-neutralize emerging variants in patient quasiespecies, and the insufficiently effective cytotoxic T-lymphocyte response to viral epitopes (Okamoto *et al.*, 1992; Jackson *et al.*, 1997; Zibert *et al.*, 1997; Shimizu *et al.*, 1994; Chang *et al.*, 1997). Attempts at protecting chimpanzees from HCV infection by inducing an immune responses to E2, and in particular to HVR1 epitopes, have been shown to be poorly effective, to a large extent in relation with the restricted specificity of the antibodies produced (Okamoto *et al.*, 1992; Farci *et al.*, 1994; Rosa *et al.*, 1996; van Doorn *et al.*, 1995). One critical limitation in the development of HCV vaccines and of passive immunotherapy has been the difficulty of eliciting cross-reactive antibodies. However, a number of investigators have pointed out that HVR1 was directly involved in the neutralization of HCV, essentially by blocking viral attachment to target cells (Okamoto *et al.*, 1992; Rosa *et al.*, 1996; Pileri *et al.*, 1998; Zibert *et al.*, 1995; Shimizu *et al.*, 1996). Preliminary data obtained in our laboratory and by others suggested that HVR1, although highly variable, retained three conserved amino acids and had substitution constraints in the mutation observed in the C-terminus part of HVR1 which is the site of neutralizing epitope (Rosa *et al.*, 1996; van Doorn *et al.*, 1995; Jackson *et al.*, 1997). We therefore formulated the hypothesis that a common, ubiquitous, HVR1 epitope might exist, related to the conserved amino acid and presumably largely conformational. To test this hypothesis, we designed immunization schemes of rabbits involving known C-terminus sequences of HVR1 derived from patients who

reacted strongly against their own HVR1 epitope and whose sequence was frequently recognized by sera from unrelated patient infected by largely divergent virus. The reactivity of two rabbits immunized with the same six HVR1 peptides injected at 2-week intervals in reverse order elicited antibodies with different specificities and levels of reactivity (Fig. 1). The sequence of immunization of rabbit 2 was considerably more effective and provided some indication that a peptide could boost antibody to another peptide less than 50% homologous. In addition, serum from each immunized rabbit reacted not only with immunizing peptides but also against unrelated peptides with limited sequence homology (Fig. 2). By mixing sera from the two rabbits an IgG preparation with larger cross-reactivity was obtained. These data suggest that an HVR1 epitope, unrelated to the linear sequence of the HVR1 peptides except for the G and Q at positions 23 and 26, was elicited by the immunization scheme. Rabbit IgG or individual rabbit sera reacted with HVR1 sequences less than 40% homologous to any of the immunizing peptides, suggesting that the binding epitope is likely to be, at least in part, conformational. These results were further supported by the cross-reactivity of peptide-immunopurified specific anti-HVR1 rabbit IgG with unrelated HVR1 peptides. Whether antibody was purified with immunizing or cross-reactive peptides, the same group of HVR1 peptides was recognized, and this recognition was independent of the peptide linear sequences. Our data strongly suggest that rabbit antibody is of restricted specificity and directed against a nonlinear epitope. Some of our data, however, are at variance with recently reported results by Esumi *et al.* (1998), who immunized mice with peptides twice as long as ours (30 aa), including the N-terminus part of HVR1. They did not observe a good immune response against the C-terminus sequences of HVR1. This difference might be related to the fact that longer peptides might conform differently from shorter ones, possibly masking critical epitopes.

Rabbit IgG has not only broad cross-reactivity with a large panel of peptides unrelated to the immunizing peptides but also high titers (Fig. 3) and high affinity together with high specificity for HVR1 (Table 2). We therefore evaluated the ability of this antibody to interact with HCV viral particles. The broad cross-reactivity and high affinity of rabbit IgG observed with HVR1 peptides was also found with whole virus as target. Sixty-five percent of HCV from random infected UK blood donors (Fig. 4) were captured by immobilized rabbit IgG. This percentage is considerably higher than the 20% (1 of 5 patients) capture observed by Esumi *et al.* (1998) using an immunoprecipitation method and antibodies obtained by immunization with peptides of considerably less sequence diversity than ours. Viral HVR1 recognition by rabbit IgG was independent of viral concentration (Table 3) and HCV genotypes, at least with genotypes 1, 2, and 3, the most prevalent in the UK. However, limited se-

quence data on captured and noncaptured HCV suggest that HVR1 linear sequences may have some relevance to the absence of capture of HCV in 35% of patient plasmas (Esumi *et al.*, 1998). This conclusion was further supported by experiments comparing rabbit IgG binding to native or denatured HVR1 peptides. After boiling, the reactivity of immunizing peptides was partially decreased, but cross-reactivity with random peptide was diversely affected. Our result suggest that rabbit IgG recognizes an epitope partly defined by the linear sequence (presumably the conserved G and Q in positions 23 and 26, respectively), partly by conformation.

An alternate explanation for the lack of capture of HCV from some patients is that only free virus may be available for antibody capture. It has been shown by several investigators that HCV circulate in plasma in either antibody-complexed or free particle forms (Hijikata *et al.*, 1993; Choo *et al.*, 1995; Aiyama *et al.*, 1996). It was therefore possible that only viruses with a sufficiently large proportion of free HCV were detectable by capture. First, the free protein G column separated HCV present in the follow-through fraction from a capture-negative plasma was not captured by the anti-HVR1 rabbit IgG, although HCV RNA was present. Second, experiments designed to determine whether IgG-complexed HCV was also captured were not totally conclusive since elution from protein G may have dissociated some virus and the ultracentrifuged pellet of HCV-infected plasma appeared contaminated with free virus. However, it seems likely that rabbit IgG can capture complexed HCV either by recognition of a different E2 epitope than that recognized by the patient antibodies or by displacement of the patient antibodies with antibodies of higher affinity. The latter hypothesis is supported by previous studies of anti-HVR1 in HCV-infected patients which showed that antibodies are of low titer (Rosa *et al.*, 1996; Jackson *et al.*, 1997) and presumably low affinity. In addition, three anti-HVR1 single chain Fv monoclonal antibodies obtained by phage display technology in our laboratory were of extremely restricted specificity and low affinity (unpublished data). In their system, Esumi *et al.* (1998) found an apparently complete immunoprecipitation of HCV from the patient whose sequences were used to derive immunizing peptides. This result only indicates that all detectable free virus was complexed with mouse HVR1 antibodies, not whether they recognized complexed viruses.

Finally, we explored the functional potential of the rabbit polyclonal anti-HVR1 by assessing its capacity to block HCV binding to susceptible cells. Several previous studies indicated that blocking viral attachment to target cells indicated neutralizing activity (Rosa *et al.*, 1996; Zibert *et al.*, 1995; Shimizu *et al.*, 1996). Rabbit IgG blocked HCV binding to MOLT-4 cells in a dose-dependent fashion but only virus in plasma from patients positive with the IgG HCV RNA capture assay. These results

are consistent with a critical role played by HVR1 in the HCV-cell interaction (Rosa *et al.*, 1996; Pileri *et al.*, 1998). In addition, total blocking of HCV binding was consistently achieved with a concentration of 5 μ g/ml of rabbit IgG. A pool of IgG from high anti-HCV-titer-infected patients blocked HCV binding to the MOLT-4 cell line at an identical concentration. These results indicate that antibodies to HVR1 raised in rabbits have the potential to block the binding of a broad spectrum of HCV strains to susceptible cells. However, blocking viral attachment is not synonymous with neutralization, which requires a culture system to be evaluated. Our results also suggest that antibodies against a widely ubiquitous HVR1 epitope can be produced and that appropriate HVR1 antigens injected in an appropriate fashion may elicit broadly cross-reactive, possibly protective, antibodies.

MATERIALS AND METHODS

Plasma

Samples of HCV-infected plasma or serum were obtained from prospective blood donors presenting at the East Anglian Blood Centre, or candidates for orthotopic liver transplantation, or asymptomatic HCV carriers referred to the consultant hepatologist at Addenbrooke's Hospital (Cambridge, UK). HCV infection was shown by the presence of antibodies using commercial HCV screening (HCV-EIA, Abbott Laboratories) and confirmatory assays (RIBA 2 Ortho Diagnostics). All were positive for HCV RNA detected by nested polymerase chain reaction (PCR) using a previously described method (Petrik *et al.*, 1997). HCV RNA was quantified as described (Lawal *et al.*, 1997).

Peptides. A series of peptides ranging in size from 16 to 19 residues corresponding to the sequence of hyper-variable region 1 and other regions of HCV structural proteins were synthesized commercially by either Cambridge Research Biochemicals Ltd. or by Severn Biotech Ltd. Peptides were obtained at a purity greater than 85%, used without further purification, were dissolved to a concentration of 4 mg/ml in dimethyl sulfoxide/H₂O (1:24, v/v) and stored at -35°C . The sequences of peptides used for immunizing and testing antibody cross-reactivity are shown in Table 1.

Generation and purification of the immune sera. Six peptides, MH2, MH3, MH4, L1.1, W1, and LV (Table 1A), were conjugated to keyhole limpet hemocyanin (KLH) (Sigma) by 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) (Sigma Chemical CO) coupling (Harlow and Lane, 1988a). Immune sera were generated by six sequential intradermal multiple site injections of the conjugated peptide in rabbits (400 μ g) in Freund's adjuvant (1, 15, 30, 45, 60, and 75 days). Rabbit 1 was injected with conjugated peptide MH2 at day 1 and subsequently injected with the other five peptides at a 2-week interval in the sequence: MH3, MH4, L1.1, W1 and LV. Rabbit 2

was injected according to the reverse sequence of conjugated peptides (LV at day 1, W1, L1.1, MH4, MH3, and MH2). Blood samples were drawn from each rabbit (pre-immunization, 45, 60, 75, and 90 days after initial injection of conjugated peptide). Sera from rabbits 1 and 2 at each time point were tested for reactivity with each immunizing peptide by ELISA. Sera at time points before and 90 days after injection from each rabbit were mixed, and the mixture was purified with a HiTrap Protein A column according to the protocol of the manufacturer (Pharmacia Biotech). The purified immune serum was designated rabbit IgG. The preimmunization IgG was used as control.

For some experiments, rabbit IgGs were further immunopurified using immobilized HVR1 peptides as ligands. HVR1 peptides were cross-linked through the added N-terminal cysteine to thiopropyl Sepharose 6B gels according to the protocol of the manufacturer (Pharmacia Biotech). Five 1.2-ml HVR1 peptide (MH3, LB.1, W1, S90, and DH1) affinity chromatography columns were prepared and used to purify antibodies against HVR1 from rabbit IgG. Each of the immobilized peptide columns was loaded with 1 ml of rabbit IgG (10 mg/ml); after washing the column with 10 ml binding buffer (20 mM sodium phosphate, pH 7.0), bound IgG was eluted with 0.1 M citric acid, pH 3.5. The collected fractions were neutralized with 1 M Tris-HCL, pH 9.0. The concentration of protein in each fraction was measured using a UV-vis spectrophotometer (Shimadzu Corp., Kyoto, Japan) and the three fractions with highest absorbance were pooled. ELISA was performed using a concentration of protein adjusted to 6.5 $\mu\text{g/ml}$.

Affinity of rabbit antibody. The affinity of rabbit IgG against HCV HVR1 peptides was determined by surface plasmon resonance using the IASYS optical biosensor (Affinity Sensor, Cambridge UK). Peptides MH2, MH3, and LV (100 $\mu\text{g/ml}$ in 10 mM sodium acetate buffer, pH 5.0) were immobilized on the surface of carboxymethyl dextran cuvettes using DEC/NHS chemistry according to the manufacturer's instructions. The remaining activated sites were blocked with 1 M ethanolamine, pH 8.5. Different concentrations of rabbit IgG in PBS were then added to the cuvettes. Association was measured for 5 min and dissociation for 3 min. Using the Fastfit program (Affinity Sensor), the apparent association (K_{on}) and dissociation (K_{off}) rates were calculated for the binding of rabbit IgG with each peptide. The K_d was then calculated as $K_{\text{off}}/K_{\text{on}}$ (Neri *et al.*, 1996).

Peptide enzyme immunosorbant assay (peptide ELISA). The reactivity of rabbit sera and rabbit IgG with various HCV peptides was determined by ELISA as follows: the wells of a 96-well microtiter plate (Nunc-immuno plate, maxisorp, Life Technologies) were coated with 100 μl per well of a 5.0 $\mu\text{g/ml}$ solution of peptides and incubated at 4°C for 16 h. The wells were washed three times with PBS and incubated for 60 min at room

temperature (22°C) with 3% BSA in PBS. Serial dilutions (100 $\mu\text{l/well}$) of rabbit sera or rabbit IgG in PBS-B buffer (4% BSA in PBS, w/v) were incubated for 60 min at 37°C and, subsequently, for 60 min at 37°C with a 100 $\mu\text{l/well}$ solution of goat anti-rabbit IgG conjugate to alkaline phosphatase (Sigma) used at a 5000-fold dilution in PBS-B. Wells were washed five times with PBS-T (0.1% Tween 20 in PBS, v/v). The alkaline phosphatase reaction was visualized using the *p*-nitrophenyl phosphate (pNPP) solution in 0.1 M glycine buffer (1 mg/ml) (200 $\mu\text{l/ml}$). After 30 min incubation at 22°C the reaction was stopped by the addition of 50 μl of 3 N sodium hydroxide to each well, and the absorbance was measured at 405 nm on a Titertech 96-well plate reader. The cutoff for each plate was calculated as the mean plus four times the standard deviation for the absorbances of 10 aliquots of preimmunization rabbit sera or IgG. In some experiments, peptides were denatured by heating at 100°C for 10 min and used to coat microtiter plates. Peptide ELISA was then performed as described.

HCV RNA capture assay. Rabbit IgG at a concentration of 1 mg/ml was labeled with biotinamidocaproate *n*-hydroxysuccinimide ester as described (Harlow and Lane, 1998b). Rabbit IgG labeled with biotin was bound to streptavidin-coated paramagnetic particles (SA-PMPs) according to the manufacturer's protocol (Promega). After washing three times, the particles were resuspended in 1 ml of PBS. SA-PMPs-IgG (cross-linked with the rabbit IgG) and SA-PMPs-plgG (cross-linked with preimmunization rabbit IgG) were used for HCV capture assay as follows: 80 μl of the SA-PMPs-IgG or SA-PMPs-plgG suspension was mixed with 150 μl of each serum or plasma (confirmed HCV RNA positive) with 1 ml of PBS in a 1.5-ml Eppendorf tube. After 2 h incubation at 37°C with rotation, the particles were concentrated with a magnetic stand (Promega). The supernatant was pipetted and used as a positive control of RT-PCR. After washing three times with 1.4 ml of PBS, an aliquot of the last wash was kept and the particles, resuspended in 150 μl PBS, were used for the extraction of HCV RNA. Preliminary studies performed in duplicate with three samples containing $1\text{--}2.5 \times 10^6$ genome equivalents of HCV RNA showed that after three washes in PBS, HCV RNA which was present in the initial microparticle supernatant after capture was no longer detectable. HCV RNA detection was performed with nested RT-PCR. In all experiments, microparticles coated with preimmunization rabbit antibody was used as negative control and patient plasma as positive control.

HCV RNA detection. Viral RNA was extracted from either 100 μl cell suspension or 100 SV-PMPs-IgG suspension using RNAzol B RNA isolation solvent according to the protocol of the manufacturer (AMS Biotechnology, Europe). The extracted RNA was resuspended in 15 μl DEPC-treated water, and 5 μl RNA was transcribed into cDNA using 30 pmol of primer 5'-GATGTACCCCATGAG-

GTCGG-3' (anti-sense primer, 732–751) by superreverse transcriptase (21 u) (HT Biotechnology Ltd., UK) in a reaction of 20 μ l. Two rounds of PCR were performed using 2 μ l of the respective cDNA mixtures in a total of 50 μ l using Ampli Taq DNA polymerase (2 μ l) and 30 pmol primers. In the first round of PCR and RT primer and the sense primer 5'-GCGCGAAGGAAGACTTCG-3' (481–499) were used. The second round of PCR was performed with primer 5'-CGTGGGAAGGCGACAACCTAT-3' (515–535) and primer 5'-CGCATGTTAGGGTATCGAT-3' (706–725). The Southern blotting analysis was carried out with probe 5'-ATGGGCTGGGCAGGATGGCTC-3' (661–632) with 5' digoxigenin-labeled using a DIG chemiluminescent kit (Boehringer Mannheim) according to the manufacturer's instructions.

Cell binding and blocking assays. The human T-cell line MOLT-4 was used in the binding and blocking assays. Cells were maintained in RPMI 1640 medium with 10% heat-inactivated fetal calf serum. To determine cell absorption efficiency of circulating HCV, plasmas MH, L1A4, L3A1, K, C, and D were serially diluted in 10-fold increments in RPMI 1640 medium, and 100 μ l of each dilution was added to 0.5 ml of a suspension of 5×10^5 MOLT-4 cells. After incubation for 2 h at 37°C, cells were washed twice with 10 ml PBS and tested for cell-adsorbed HCV RNA by RT-PCR using nested primers which detected the core region of HCV genome. This series of experiments provided for each sample a HCV RNA absorption titer which was subsequently used to adjust the virus concentration in blocking experiments. The blocking assay was as follows: a 100- μ l sample of each target virus (at the adjusted dilution of 10^{-2} for sample L3A1; 10^{-1} for L1A4, C, and D; undiluted for MH and K) was incubated overnight at 4°C with an equal volume of either rabbit IgG (5 μ g/ml, 0.5 μ g/ml, 50 ng/ml, and 5 ng/ml) or control preimmunization IgG. Each mixture was added to 0.5 ml of a 5×10^5 MOLT-4 cell suspension and incubated for 2 h at 37°C. After washing twice with 10 ml of PBS, RNA was extracted and HCV RNA in cell pellets and supernatants was detected by RT-PCR. After the first wash with PBS, HCV RNA was no longer detectable. All experiments were carried out in duplicate. The positive control was patient plasma supernatant after cell absorption and the negative control was plasma of a HCV RNA-negative blood donor.

ACKNOWLEDGMENTS

This work was supported by a grant from the University of Cambridge, UK. Dr Zhai was supported by Grant 95/7 from the National Blood Authority. The authors thank Dr. P. Jackson for his technical help with the peptide assays.

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